Studies on the effect of β-caryophyllene on insulin signaling molecules in Gastrocnemius muscle of high fat diet and fructose induced type-2 diabetic experimental rats

BACKGROUND

Type 2 diabetes mellitus (T2DM) is one of the very common metabolic disorder and is associated with co-morbidities, such as obesity, hyperlipidemia (increased VLDL which taken together, comprise the 'Metabolic Syndrome' (Kaur *et al*., 2014). It causes significant morbidity and mortality at considerable expense to patients, their families and society. International Diabetes Federation (IDF) 2019 report is stating that around 463 million people worldwide are suffering from this disease and the prevalence is predicted to cross the figure of 700 million by the year 2045. Asia is a major area of emerging T2DM global epidemic, with China and India the top two epicenters. Although genetic predisposition partly determines individual susceptibility to T_2DM, an unhealthy diet and a modern lifestyle are important drivers of the current global epidemic; early developmental factors (such as intrauterine exposures) also have a role in susceptibility to T_2DM later in life (IDF, 2019; Zheng *et al*., 2017).

It is characterized by relative insulin insufficiency results in hyperglycemia (Mahler and Adler, 1999) and insulin resistance (DeFronzo, 2004) and the cause can be localized in part to a defect in glucose transport. The three major tissues muscle, liver and adipose tissue largely responsible for clearing glucose from the blood in healthy individuals become insulin resistant in T2DM (DeFronzo et al., 1985) Adipose tissue, which is a metabolically dynamic organ, plays a vital role in the metabolism of glucose and lipid and in the secretion of large number of cytokines and hormones (Coelho et al., 2013). It has been shown that excessive

intake of high-fat diet ends up in fatness which might induce insulin resistance in target tissues. The fatty tissues within the rotund state are mostly expanded and performance abnormally within the regulation of cytokine release, fatty acid metabolism and its storage that results in insulin resistance (McArdle et al. 2013). Insulin signaling is impaired during insulin resistance condition at multiple levels, leading to reduced glucose uptake in insulin-sensitive peripheral tissues (Kahn and Flier, 2000)

Insulin-regulated disposal of glucose from the bloodstream into skeletal muscle and fat tissues is mediated by the glucose transporter GLUT4, which undergoes translocation from intracellular storage sites to the cell surface upon insulin stimulation (Wang et al., 2013). Insulin binding to its receptor, results in autophosphorylation of a number of tyrosine residues in the receptor (White and Kahn, 1994; Cheatham and Kahn, 1995). Further, it recruits the insulin receptor substrate (IRS) family of proteins. Tyrosine-phosphorylated IRS display binding sites for phosphatidylinositol-3 kinase (PI3K), which, in turn, activates Akt/protein kinase B (Manning, 2010), The insulin-activated PI3K/PKB (protein kinase B, also known as Akt) signalling axis plays a key role in mediating insulinstimulated GLUT4 trafficking (Huang and Czech, 2007). Any alteration in this signalling pathway leads to failure of or improper glucose uptake which ultimately results in impaired glucose homeostasis and T2DM. Hunting for substrate(s) 2 downstream of PKB in controlling GLUT4 trafficking has been the focus of research in the field for decades. The decreased insulin-stimulated glucose uptake is due to impaired insulin signalling and multiple post-receptor intracellular defects including impaired phosphorylation, translocation of glucose transporter, reduced glucose oxidation and glycogen synthesis (Bajaj and Defronzo, 2003; Karlsson and Zierath, 2007).

T2DM is associated with substantial morbidity and mortality (Kishida et al., 2012). Management of diabetes is complex and requires marked effort from the people with diabetes to achieve recommended self-management behaviors. At present, therapy for T2DM relies mainly on several approaches intended to reduce the hyperglycaemia itself: sulphonylureas (and related insulin secretagogues), metformin, peroxisome proliferator-activated receptor-γ (PPARγ) agonists (thiazolidinediones), which enhance insulin action; α-glucosidase inhibitors, which interfere with gut glucose absorption (Malvi et al., 2011). The need for new therapies for glycemic control is the fact that the commercially available antidiabetic drugs are associated with side effects affecting blood lipid levels and/or systemic blood pressure, limited efficacy, and tolerability and significant mechanism-based side effects which may lead to increase in morbidity and mortality (Aguilar, 2011; Malvi et al., 2011; Prabhakar et al., 2014). Despite the impressive advances in health sciences and medical care, there are many patients who are using alternative therapies alone or complementary to the prescribed medication (Alexiou and Demopoulos, 2010). There is a growing interest in herbal remedies because of their effectiveness, minimal side effects and relatively low cost (Singh et al., 2012). Several species of medicinal plants are used in the treatment of diabetes mellitus (Singh et al., 2012).

β-Carophyllene is a natural sequiterpene, widely present in cannabis as well as many culinary herbs and spices. Black pepper, cloves, cinnamon, hops, rosemary and hemp are good sources of this terpene. It has a many biological effects such as antioxidant, anti-inflammatory and anti-lipidemic effects (Chen et al., 2019). It could help to reduce cholesterol, relieve pain and anxiety and treat seizures. β-Carophyllene exhibits a protective effects against oxidative stress as well as a being anti-inflammatory, it could be an effective preventative medicine against a various medical conditions such as liver diseases, kidney diseases, liver and gastrointestinal

diseases, immune and neurodegenerative diseases (Bento et al., 2011; Calleja et al., 2013; Horvath et al., 2012; Chen et al., 22019).

Basha and Sangaranarayanan (2014) elucidated that administration of β-Caryophyllene has potential effects on the regulation of glucose homeostasis in diabetic rats by regulating carbohydrate metabolism. It inhibits the activity of intestinal α-glucosidase which is involved in disaccharide metabolism, an attractive drug target in diabetes treatment, and it could be used as first line drug or defense to control hyperglycemia. Aldose reductase is another effective drug target which is responsible for the accumulation of sorbital, another consequence of hyperglycemia. Glycosylation of proteins is another unavoidable consequence of prolonged hyperglycemia, which is formed by non-enzymatic reactions resulting to the formation of advanced glycation end products (AGEs), can induce various complications of diabetes such as cardiovascular diseases, stroke and Alzheimer's disease (Chen et al., 2019).

Chronic oral administration of β-Caryophyllene reduces glyceamia, depressive-like behavior and neuropathic pain in streptozotocin (STZ)-induced diabetic mice (Aguilar-Ávila et al., 2019). In addition, recently, it has been elucidated that β-Caryophyllene effectively protects β-cells by alleviating hyperglycemia through increasing insulin release, and also ameliorate oxidative stress and inflammation in pancreatic tissue of experimental diabetic rats (Basha and Sankaranarayanan, 2016). These promising reports manifest the medicinal properties of β-Caryophyllene. Although β-Caryophyllene has been shown to have antidiabetic effects, the mechanism by which it exhibits antidiabetic effect is not fully known. Therefore, the present study was designed to appraise the molecular mechanism underlying the antidiabetic effect of β-Caryophyllene.

HYPOTHESIS

β-caryophyllenemay exhibit antidiabetic effects through regulating insulin signaling molecules and also by the biochemical profiles in type-2 diabetic rats

AIM

The present study was aimed to identify the molecular role of β-caryophyllene on insulin signaling molecules in skeletal muscle of high fat diet and fructose –induced type-2 diabetic experimental rats.

OBJECTIVES

- To study the protective role ofβ-caryophyllene on fasting blood glucose, Glucose and insulin tolerances, serum insulin and testosterone, HOMA- IR, QUICKI, liver and kidney functions marker, serum lipid profile, oxidative stress markers and antioxidant enzymes in experimental rats
- To identify the effects of β- caryophyllene on Insulin signaling molecules (IR, IRS-1,Akt, and GLUT4 proteins), glycogen content, glucose uptake and oxidation in skeletal Muscle of type-2 diabetic experimental rats.

MATERIALS AND METHODS

Animals

Healthy adult male Wistar rats (180–200 g body weight) (24 animals)were obtained from the Central Animal House Facility, Meenakshi Medical College and Research Institute (Meenakhsi Academy of Higher Education and Research), Enathur, Kanchipuram, Tamil Nadu, India. Animals were maintained as per the

guidelines and protocols approved by the Institutional Animal Ethics Committee and by the regulatory body of the government (IAEC No: 007/2019, dated 04/11/2019) under laboratory conditions of temperature (22±21˚C), humidity (45±5%) and 12 h day: 12 h night cycle, and were allowed free access to food (standard pellet diet, SaiDurga Feeds and Foods, Bangalore, India) and water *ad libitum*.

Induction of Type-2 Diabetes

Rats were made diabetic (type-2) by feeding high fat diet containing 2%cholesterol, 1%cholic acid, 30%coconut oil, 67% standard rat feed, and 25% Fructose through drinking water for 60 days (Sampath et al., 2013; Augustine et al., 2014). After 60 days, fasting blood glucose level was tested and animals with >120 mg/dl blood glucose were selected for the experiment. High fat diet and fructose feeding were continued till the end of study. Control rats were fed with normal pelleted rat feed and water was made available *ad libitum.*

Dose fixation study:

The optimal dose of β-Carophyllene will be fixed on the basis of the following dose fixation study. Rats will randomly be divided into the following groups of three each.

Group I	Normal control rats (1ml/kg saline);
Group II	Rats are induced with diabetes by a high fat-fructose diet containing cholesterol 2 g, cholic acid 1 g, coconut oil 30 mL, standard rat feed 67 g, and 25% fructose through drinking water and kept without any treatment for 60 days.
Group III	Rats are induced with diabetes as mentioned in group 2 and treated with β-Carophyllene(50mg/kg body weight once a day, orally) for 30 days, respectively.

Group IV	Rats are induced with diabetes as mentioned in group 2 and treated with β-Carophyllene(100mg/kg body weight once a day, orally) for 30 days, respectively.
Group V	Rats are induced with diabetes as mentioned in group 2 and treated with β-Carophyllene(200mg/kg body weight once a day, orally) for 30 days, respectively.
Group VI	Rats are induced with diabetes as mentioned in group 2 and treated with β-Carophyllene(400mg/kg body weight once a day, orally) for 30 days, respectively.

Experimental design

The following experimental design was framed and accordingly the rats were subjected to treatment for a period of one month. Healthy adult male Wistar rats were divided into the following groups of 6 rats each.

Group I: Control (Normal rats).

Group II: Rats were made diabetic (type-2) after feeding high fat diet & fructose through drinking water (30%) for 60 days.

Group III: Type-2 diabetic rats treated orally with β-caryophyllene (50 mg/kg b.wt/day) for 30 days

Group IV: Type-2 diabetic rats treated orally with metformin (50 mg/kg, b.wt/ day for 30 days

Group V: Control rats administered orally with β-caryophyllene (50 mg/kg b.wt/day) for 30 days.

Two days prior to kill, control and experimental animals were subjected to oral glucose tolerance test (OGTT) and insulin tolerance test (ITT). At the end of 30 days, blood was collected and animals were perfused with physiological saline

under sodium thiopentone (40 mg/ kg, b.wt) anesthesia. blood was collected through cardiac puncture, sera were separated and stored at −80°C, and skeletal muscle was dissected out for the assessment of various parameters.

Fasting blood glucose (FBG):

Blood glucose was estimated using On-Call Plus blood glucose test strips (ACON Laboratories Inc., USA) after overnight fasting. Blood was collected by pricking the tip of the rat tail and results are expressed as mg/dl.

Oral Glucose Tolerance Test (OGTT):

For oral glucose tolerance test, animals were fasted overnight and blood glucose was estimated using On-Call Plus blood glucose test strips at various time periods (60, 120 and 180 min) after giving the oral glucose load (10 ml/kg; 50% w/v). Blood glucose value before giving glucose load is considered as 0 minute value. Results are expressed as mg/dl.

Insulin Tolerance Test (ITT):

This test was performed two days before sacrificing the animals. Rats were injected with insulin (0.75 U/kg) in ~ 0.1 ml 0.9% saline intraperitoneally. A drop of blood (5 µl) was taken from the tail vein before the injection of insulin (considered as "0" h reading of blood glucose) and after 15, 30, 45, and 60 min for the determination of blood glucose with a glucometer. Results are expressed as mg/dl.

Fasting serum insulin:

This test was performed using ultrasensitive rat insulin ELISA kit obtained from Crystal ChemInc (Illinois, USA). The range of detection is 0.1 - 64 ng/ml. The percentage cross-reactivity of insulin antibody to rat insulin was 100%. Intra-assay coefficient of variation was ≤10.0% and inter-assay coefficient of variation was ≤10.0%. Results are expressed as ng/ml.

Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) and Quantitative Insulin Sensitivity Check Index (QUICKI)

HOMA-IR was calculated using the formula (fasting blood glucose X fasting serum insulin/405) based on the method of Matthews et al. (1985) and QUICKI was calculated according to the method of Katz et al. (2000) by using the formula 1/ (log fasting serum insulin + log fasting blood glucose).

Serum lipid profile

Free fatty acid (FFA), Serum cholesterol (CHO), triglyceride (TG), low-density lipoproteins (LDL) and High-density lipoproteins (HDL) were assessed using assay kits procured from Spinreact, Spain. Results for same are marked as mg/dl.

Liver and kidney function markers

Liver function markers (ALT, AST and ALP) and kidney function markers (urea and creatinine) were assessed using biochemical-assay kits purchased from Spinreact, Spain. Results for same were expressed as U/L.

Oxidative Stress markers in Skeletal Muscle

1. ***Lipid peroxidation and reactive oxygen species***

Lipid peroxidation (LPO) was measured by the method of Devasagayam and Tarachand, (1987). The malondialdehyde (MDA) content of the sample is expressed as nmoles of MDA formed/min/mg protein. Hydrogen peroxide generation was assessed by the spectrophotometric method of Pick and Keisari, (1981) and expressed as μmoles/min/mg protein. Hydroxyl radical (OH*) production was quantified by the method of Puntarulo and Cederbaum, (1988) and expressed as μmoles/min/mg protein.

2. ***Protein carbonyls Assay***

Protein carbonyls were measured according to procedure described by Reznick and Packer (1994) using dinitrophenylhydrazine (DNPH) reagent and spectrophotometric method.The absorbance was measured at 370 nm. The results were expressed as nanomoles of carbonyl groups per milligram of protein using a molar extinction coefficient of 22,000 M-1 cm-1.

Skeletal Muscle Antioxidant enzymes

The superoxide dismutase (SOD) was assessed by the method of Marklund and Marklund (1974) and the results are expressed in units/mg protein. Catalase activity (CAT) was measured as per the method of Sinha (1972) and the results for

which are expressed as units/mg protein. Glutathione peroxidase (GPx) levels were analysed by method of Rotruck et al. (1973) and the activity are expressed as mg of glutathione utilized/min/mg protein respectively.

Skeletal Muscle 14C-2-deoxyglucose uptake

Glucose uptake was estimated using 14C-2-dexoyglucose as per the method of Nevado et al. (2006). Results are notified as counts per minute (CPM) of 14C-deoxyglucose uptake/ 100 mg tissue.

Skeletal Muscle 14C-glucose oxidation

Glucose oxidation was estimated using 14C-glucose as per the method of Muthuswamy et al. (2007). Results are marked as CPM of 14CO2 released/100 mg tissue.

Skeletal Muscle Glycogen content

Glycogen was estimated by the method of Hassid and Abraham (1957). Results for the amount of glycogen concentration are mentioned as mg/g wet tissue.

Gene expression analysis in Skeletal Muscle

Isolation of total RNA, cDNA conversion and real-time PCR

Total RNA was isolated by the method explained by Fourney et al. (1988), from gastrocnemius muscle of control and experimental sample using a TRIR kit (Total RNA Isolation Reagent). Briefly, using 1 ml of TRIR, 100 mg fresh tissue

was homogenized. To this content, 0.2 ml of chloroform was added, mixed by vortexing and kept at 4^{e} C for 5 minutes. After centrifugation of the content at 12,000xg for 15 minutes at 4^{e}C, aqueous phase (upper layer) was charily shifted to a fresh microfuge tube and equal volume of isopropanol was added and mixed by vortexing for15 seconds and kept at 4^{e} C for 10 minutes. Again the contents were centrifuged at 12000g for 10 minutes at 4^{e} C, the supernatant was discarded and RNA pellet was washed with 1ml of 75% ethanol by vortexing. The concentration of RNA was determined spectrophotometrically and the results are expressed in microgram (µg).

According to manufacturer's protocol, the complementary DNA was synthesized from 2 mg of total RNAby using the reverse transcriptase RT kit from Eurogentec (Seraing, Belgium). To perform Real- Time PCR, all the elements [2x reaction buffer (Takarsybr green mastermix), β-actin, forward and reverse primers of the target gene & house-keeping gene and water in total volume of 45 µl were mixed completely an spun down. About 5 µl of control DNA for positive control, 5 µl of water for negative control and 5 µl of template complementary DNA for samples were occupied in separate PCR vials and 45 µl of reaction mixture was added into the reaction bottle and set up for 40 cycles (95℃ for 5 min, 95℃ for 5 s, 60℃ for 20 s and 72℃ for 40 s). The PCR machine (Stratagene MX 3000P, Agilent Technologies, 530l, Stevens Creek Blvd, Santa clara CA, 95051) was plotted the obtained results on a graph; relative quantification was calculated by analyzing the obtained amplification curve and melt curve. The qualified amount of mRNA was calculated with help of comparative method.

Protein expression analysis in Skeletal Muscle

Isolation of protein

For protein isolation, 100mg of tissues from control and treated animals were homogenized by using buffer A (10 mM NaHCO3, 0.25 M sucrose, 5 mM NaN_3) and centrifuged at 1300xg for 10 min at 4°C. Then the supernatant was centrifuged for 15 minutes at 12,000xg in 4°C. The resultant supernatant was used for the analysis of post-receptor insulin signaling molecules (IR, IRS-1,Akt,and GLUT4 (measured in both cytosolic and plasma membrane fractions)and the concentration of protein was identified using BSA (bovine serum albumin) as a standard by the method of Lowry et al. (1951).

Western blot analysis of proteins

The lysate proteins (50 µg/ lane) were separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (10% gel) and transferred by electroblotting to polyvinylidenedifluoride (PVDF) membrane (Bio-Rad Laboratories, USA). The membranes were blocked with 5% nonfat dry milk and probed with the primary antibodies (which were diluted to 1:1000). Following incubation, the blot was washed 9 for three times (5 min each with Tris-buffered saline containing Tween-20 (TBS-T)). After washing with TBS-T, membranes were incubated for 1 hour with horseradish peroxidase-conjugated rabbit-anti-mouse or goat-anti-rabbit antibodies (which were diluted to 1:5000) (GeNei, Bangalore, India). The specific signals were detected with an enhancedchemiluminescence detection system (Thermo Fisher scientific Inc, USA). The protein bands were

captured using Chemidoc and quantified by Quantity One image analysis system, Bio-Rad Laboratories, USA. Later the membranes were incubated in stripping buffer at 50°C for 30 min. After this, the membranes were reprobed using a β-actin antibody (1:5000). As the invariant control, the present study used rat β-actin.

STATISTICAL ANALYSIS

The datas obtained were subjected to statistical analysis using one-way analysis of variance (ANOVA) and Duncan's multiple range test to assess the significance of individual variations between the control and treatment groups using a computer based software (Graph Pad Prism version 5). In Duncan's test, the significance was considered at the level of $p<0.05$.

RESULT

The result will be discussed later

CONCLUSION

It is concluded from the present findings that, the antidiabetic effect of β-Carophyllene in high fat diet and fructose -induced type-2 diabetic ratsis mediated throughamelioration of oxidative stress & dyslipidemia, improved insulin signal transductionand GLUT4 expression as well as glucose uptake and oxidation in gastrocnemius Muscle. Hence, β-Carophyllene can be served as one of the therapeutic phytomedicines for the management of type-2 diabetes mellitus.

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